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14. ABSTRACT: This report results from a contract tasking IMAR-Mitochondrial Research Group as follows: The main toxicological concern is the fact that some of the manufactured nanomaterials are transported across cell membranes, especially into mitochondria (Foley et al., 2002). Nanoparticles-induced mitochondrial perturbation has important biologic effects, which include the initiation of apoptosis and decreased ATP production (Hiura et al. 2000). Mitochondrial damage is a key event in particulate matter (PM) induced cytotoxicity (Hiura et al. 1999, 2000). The initial response to PM is a decrease in mitochondrial membrane potential and increased $O_2^{\cdot-}$ production, followed by cytochrome c release and inner mitochondrial membrane damage (Hiura et al. 1999, 2000; Upadhyay et al. 2003). A more recent study, showed that in BRL3A cells, nanoparticles lead to cellular morphological modifications, LDH leakage and mitochondrial dysfunction, in particular, cause increased generation of ROS, depletion of GSH, and reduction of mitochondrial membrane potential (Hussain et al, 2005). Since mitochondria are provided with a variety of bioenergetic functions mandatory for the regulation of intracellular aerobic energy production and electrolyte homeostasis, impairment of mitochondrial function by nanoparticles may have drastic consequences on cellular function through the perturbation of the bioenergetic charge and balance of the cell. The mitochondrial inner membrane can undergo a permeability increase specifically inhibited by the immuno-supressive agent Cyclosporine A (Cy A) (Broekemeier et al., 1989). This transition is manifested by the transformation of a calcium-dependent, thiol-regulated, voltage-gated complex of membrane-spanning proteins, into a nonspecific pore capable of conducting solutes of <1500Da (Zoratti and Szabo, 1995). Induction of the permeability transition pore (PTP) is widely implicated in the mechanism by which many agents interfere with mitochondrial bioenergetics in vitro (Bernardi et al., 1994; Gunter et al., 1994). Experimentally, the permeability transition is characterized by an abrupt swelling and depolarization of the mitochondria, reflecting the loss of ability to maintain ion and solute gradients across the inner membrane. The result is inhibition of oxidative phosphorylation, bioenergetic deficits, and, presumably, cell death. The results from the project demonstrate that silver nanoparticles cause impairment of mitochondrial function, due mainly to alterations of mitochondrial membrane permeability that reflect an uncoupling effect on the oxidative phosporylation system, being toxicity dependent on particle size. Thus, mitochondrial toxicity may have a centrol role in the toxicity caused by exposure to silver nanoparticles.

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TABLE OF CONTENTS

SUMMARY

Nanomaterials, which range in size from 1 to 100 nm, have been used to create unique devices at the nanoscale level possessing novel physical and chemical functional properties. The major toxicological concern is the fact that some of the manufactured nanomaterials are redox active, and some particles transport across cell membranes and especially into mitochondria. As so, evaluate their toxicity upon acute exposure is extremely necessary. In this work, we evaluated the toxicity of silver nanoparticles (40nm and 80nm) and their effects in rat liver mitochondria bioenergetics.

Wistar rat liver mitochondria demonstrate alterations in the mitochondrial respiration and membrane potential capacities, in the presence of both sizes of silver nanoparticles. Our data demonstrated statistically significant decrease in the mitochondrial membrane potential, ADP-induced depolarization, and Respiratory Control Ratio (RCR). Moreover, there is a clear decreased in repolarization and state 3 respiration, while lag phase and state 4 respiration are increased. Mitochondrial permeability transition (MPT), involved in several vital cellular signalling pathways was evaluated through mitochondrial swelling assays. The data indicate an increase in mitochondrial calcium sensitivity, with consequent mitochondrial permeability transition pore induction, in the presence of the nanoparticles (especially 80nm size). Neither mitochondrial calcium fluxes and reactive oxygen species production are affected, as well as respiratory chain complexes activities and adenine nucleotide content.

Our results show that silver nanoparticles cause impairment of mitochondrial function, due mainly to alterations of mitochondrial membrane permeability that reflect an uncoupling effect on the oxidative phosphorylation system, being toxicity dependent on particle size. Thus, mitochondrial toxicity may have a central role in the toxicity caused by exposure to silver nanoparticles.

INTRODUCTION

Nanomaterials, which range in size from 1 to 100 nm, have been used to create new types of analytical tools for biotechonology and life sciences (Cui et al., 2001). Despite its wide application, there is a serious lack of information concerning their impact on human health and the environment due to the limited number of studies available on toxicity of nanoparticles for risk assessment. In vivo exposure to nanoparticles is likely to have potential impact on the liver since exposure to these particles is likely to occur through ingestion and clearance by the liver.

The main toxicological concern is the fact that some of the manufactured nanomaterials are transported across cell membranes, especially into mitochondria (Foley et al., 2002). Although, it is still a matter of debate whether nanomaterials target the mitochondrion directly or enter the organelle secondary to oxidative damage (Li et al. 2003), nanoparticles-induced mitochondrial perturbation has important biologic effects, which include the initiation of apoptosis and decreased ATP production (Hiura et al. 2000). Mitochondrial damage is a key event in particulate matter (PM) induced cytotoxicity (Hiura et al. 1999, 2000). The initial response to PM is a decrease in mitochondrial membrane potential and increased O2·– production, followed by cytochrome *c* release and inner mitochondrial membrane damage (Hiura et al. 1999, 2000; Upadhyay et al. 2003). A more recent study, showed that in BRL3A cells, nanoparticles lead to cellular morphological modifications, LDH leakage and mitochondrial dysfunction, in particular, cause increased generation of ROS, depletion of GSH, and reduction of mitochondrial membrane potential (Hussain et al, 2005).

Since mitochondria are provided with a variety of bioenergetic functions mandatory for the regulation of intracellular aerobic energy production and electrolyte homeostasis, impairment of mitochondrial function by nanoparticles may have drastic consequences on cellular function through the perturbation of

the bioenergetic charge and balance of the cell. The mitochondrial inner membrane can undergo a permeability increase specifically inhibited by the immuno-supressive agent Cyclosporine A (Cy A) (Broekemeier *et al.,* 1989). This transition is manifested by the transformation of a calcium-dependent, thiol-regulated, voltage-gated complex of membrane-spanning proteins, into a nonspecific pore capable of conducting solutes of <1500Da (Zoratti and Szabo, 1995). Induction of the permeability transition pore (PTP) is widely implicated in the mechanism by which many agents interfere with mitochondrial bioenergetics in vitro (Bernardi *et al.,* 1994; Gunter *et al.,* 1994). Experimentally, the permeability transition is characterized by an abrupt swelling and depolarization of the mitochondria, reflecting the loss of ability to maintain ion and solute gradients across the inner membrane. The result is inhibition of oxidative phosphorylation, bioenergetic deficits, and, presumably, cell death.

METHODS

Isolation of liver mitochondria. Liver mitochondria was isolated from Wistar and GK rats by conventional methods (Gazotti *et al.*, 1979) with slight modifications. Protein was determined by the biuret method calibrated with bovine serum albumin (Gornall *et al.*, 1949).

Mitochondrial respiration. Oxygen consumption of isolated mitochondria was monitored polarographically with a Clark oxygen electrode (Estabrook, 1967) connected to a suitable recorder in a 1 ml thermostated water-jacketed closed chamber with magnetic stirring, at 25° C.

Mitochondrial membrane potential measurements. The mitochondrial transmembrane potential was estimated by calculating the transmembrane distribution of TPP^+ (tetraphenylphosphonium) with a TPP^+-selective electrode prepared according to Kamo *et al.* (Kamo *et al.*, 1979) using a calomel electrode as the reference.

Enzymatic activities (from the respiratory chain). Succinate dehydrogenase activity was measured polarographically (Singer, 1974). Cytochrome c oxidase activity was measured polarographically (Brautigan *et al.*, 1978). Succinate-cytochrome c reductase activity was measured spectrophotometrically (Tisdale, 1967), by following the reduction of oxidized cytochrome c as an increase in absorbance at 550 nm. ATPase activity was determined by monitoring the pH change associated with ATP hydrolysis (Madeira et al., 1974).

Determination of mitochondrial adenine nucleotides content. Adenine nucleotides (ATP, ADP and AMP) were extracted using an alkaline extraction procedure and were separated by reversed-phase high-performance liquid chromatography, as described by Stocchi (Stocchi et al., 1985).

*Measurement of the mitochondrial permeability transition***.** Mitochondrial swelling was estimated by changes in light scattering as monitored spectrophotometrically at 540 nm. The reaction medium was stirred continuously and the temperature maintained at 25°C. The experiments were started by the addition of 1 mg of mitochondria to a final volume of 2 ml of the standard incubation medium, supplemented with 3 μM rotenone, 0.5 μg oligomycin and 5 mM succinate (Palmeira and Wallace, 1997).

Determination of mitochondrial superoxide production. Mitochondrial ROS production were determined with the fluorogenic probe MitoSOXRed (Invitrogen). Mitochondria were loaded with 5 μM of MitoSOXRed for ten minutes at 37º C and then washed with PBS. The fluorescence was determined at 510 nm (excitation) and 580 nm (emission). Rotenone was included as a positive control.

Measurement of mitochondrial calcium fluxes. The accumulation and release of calcium by isolated mitochondria was determined using a calcium-sensitive fluorescent dye, Calcium Green-5 N, as previously described. The reactions were carried out at 25°C, in 2 ml of the standard incubation medium, supplemented with 3 μM rotenone and 0.4 μg oligomycin. Free calcium was monitored with 100 nM Calcium Green-5 N. Mitochondria (0.4 mg) was suspended with constant stirring in a water-jacketed cuvette holder. Fluorescence was recorded continuously using a fluorescence spectrometer with excitation and emission wavelengths of 506 and 531 nm, respectively. Energization was obtained with 5 mM succinate. The calibration was made with known amounts of calcium.

RESULTS

Effects of silver nanoparticles of diferent sizes in mitochondrial transmembrane potential

Taking into account the fundamental role of mitochondrial transmembrane potential for the phenomenon of oxidative phosphorylation, $\Delta\Psi$ was evaluated in succinate-energized mitochondria (Fig. 1). $\Delta\Psi$ was decreased in mitochondria pre-incubated with both sizes (40 and 80 nm) of silver nanoparticles.

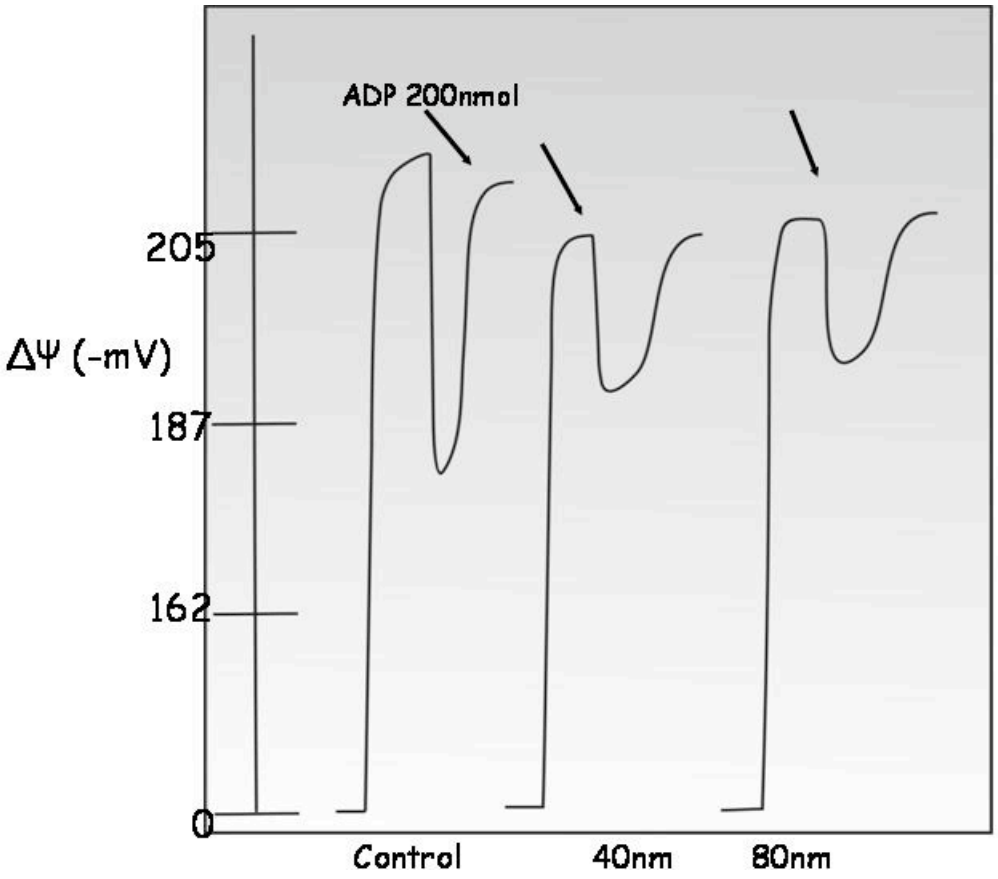

Fig. 1. Mitochondrial transmembrane potential ($\Delta\Psi$) in succinate-energized liver mitochondria, upon incubation with silver nanoparticles. $\Delta\Psi$ was measured with a TPP^+-selective electrode. Reactions were carried out in 1 ml of reaction medium,

supplemented with 2 μM rotenone and 1 mg of freshly isolated mitochondria, as described in materials and methods. Energization was achieved with 5 mM succinate and phosphorylation induced by 200 nmol ADP. The traces represent typical direct recordings of experiments performed with 10 different mitochondrial preparations.

ADP-induced depolarization and $\Delta\Psi$ after repolarization (mitochondrial capacity to establish $\Delta\Psi$ after ADP phosphorylation) was also decreased in mitochondria pre-incubated with with both sizes (40 and 80 nm) of silver nanoparticles (Table 1).

Table 1. Mitochondrial membrane potential ($\Delta\Psi$) determined in the presence of succinate as respiratory substrate, after incubation with silver nanoparticles

		40nm		80nm	
	Control	2μg/mg protein	5μg/mg protein	2μg/mg protein	5μg/mg protein
Initial ΔΨ (-mV)	211.30 ± 2.7	207.50 ± 2.6*	206.30 ± 2.1*	207.00 ± 3.3*	207.8 ± 2.6*
ΔΨ Depolarization (-mV)	28.54 ± 1.4	18.69 ± 2.6*	18.87 ± 3.2*	17.89 ± 2.9*	18.59 ± 3.1*
ΔΨ Repolarization (-mV)	209.7 ± 2.4	207.60 ± 2.2	206.80 ± 1.9	206.90 ± 2.6	207.40 ± 2.1*

Reactions were carried out in 1 ml of reaction medium, supplemented with 2 μM rotenone and 1 mg of freshly isolated mitochondria, as described in materials and methods. Data are means ± S.E.M of experiments performed with 10 different mitochondrial preparations. * indicates statistically significant difference versus control (P<0.05).

The lag phase (time necessary for ADP phosphorylation) was significantly enlarged when mitochondria was incubated with both sizes (40 and 80 nm) of silver nanoparticles (Fig. 2).

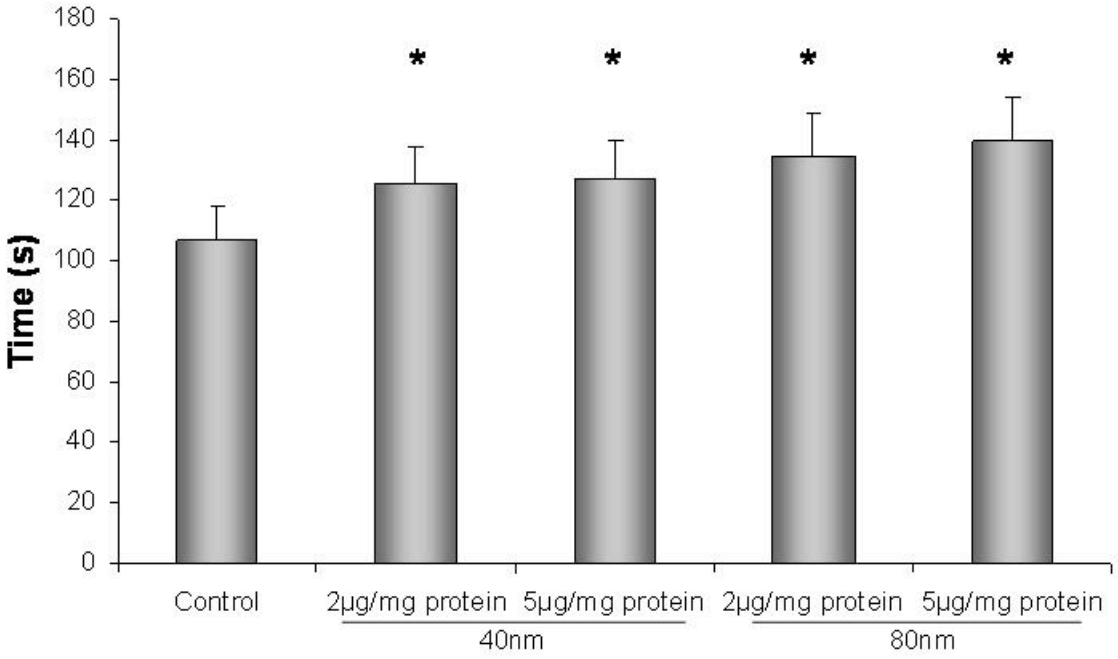

Fig. 2. Lag phase in succinate-energized liver mitochondria, upon incubation with silver nanoparticles. $\Delta\Psi$ was measured with a TPP^+-selective electrode. Reactions were carried out in 1 ml of reaction medium, supplemented with 2 μM rotenone and 1 mg of freshly isolated mitochondria, as described in materials and methods. Data are means ±

S.E.M of experiments performed with 10 different mitochondrial preparations. * indicates statistically significant difference versus control (P<0.05).

Effects of silver nanoparticles of diferent sizes in mitochondrial respiration

Oxidative phosphorylation capacity was investigated by following oxygen consumption upon succinate oxidation. Mitochondrial state 3 respiration (ADP-induced oxygen consumption) was statistically significant decreased in mitochondria pre-incubated with both sizes (40 and 80 nm) of silver nanoparticles (Table 2). Oxygen consumption stimulated by FCCP, a well known respiratory chain uncoupler, was not affected.

Table 2. State 3 respiration, state 4 respiration, respiratory control ratio (RCR) and FCCP-stimulated oxygen consumption (V FCCP) in liver mitochondria, upon incubation with silver nanoparticles.

		40nm		80nm	
	Control	2μg/mg protein	5μg/mg protein	2μg/mg protein	5μg/mg protein
State 3 (natoms O/min/mg prot)	105.3 ± 11.8	88.06 ± 13.01	77.44 ± 15.41	84.22 ± 13.0	88.23 ± 14.0
State 4 (natoms O/min/mg prot)	18.84 ± 1.7	24.58 ± 5.4	21.50 ± 5.1	25.4 ± 4.6	20.48 ± 4.1
RCR	5.79 ± 0.9	3.88 ± 0.7*	4.07 ± 0.3	3.71 ± 0.8	4.95 ± 2.1
FCCP (natoms O/min/mg prot)	209.3 ± 16.1	207.30 ± 11.3	200.70 ± 12.3	189.60 ± 18.9	194.60 ± 10.2

Reactions were carried out in 1.4 ml of reaction medium, supplemented with 2 μM rotenone and 1 mg of freshly isolated mitochondria, as described in materials and methods. Energization was achieved with 5 mM succinate and phosphorylation induced by 200 nmol ADP. Data are means ± S.E.M of 10 different mitochondrial preparations. * indicates statistically significant difference versus control (P<0.05).

The consumption of oxygen after ADP phosphorylation (state 4 respiration) was increased in mitochondria pre-incubated with both sizes (40 and 80 nm) of silver nanoparticles, when compared with control (Table 2). Consequently, the ratio between mitochondrial state 3 and state 4 respiration (RCR), was decreased in mitochondria pre-incubated with both sizes (40 and 80 nm) of silver nanoparticles (Table 2), suggesting an uncoupling effect of these nanoparticles.

Effects of silver nanoparticles of diferent sizes in mitochondrial ATPase activity, succinate dehydrogenase and cytochrome c oxidase activities

Both sizes (40 and 80 nm) of silver nanoparticles, showed no effect on the activities of these respiratory complexes, as well as on the ATPase activity of liver mitochondria (data not shown).

Effects of silver nanoparticles of diferent sizes on mitochondrial adenine nucleotides content

Both sizes (40 and 80 nm) of silver nanoparticles, do not affect mitochondrial levels of ATP, ADP and AMP (data not shown).

Effects of silver nanoparticles of diferent sizes on the induction of the mitochondrial permeability transition (MPT)

Since mitochondria possess a finite capacity for accumulating calcium before undergoing the MPT, calcium-induced mitochondrial swelling was evaluated. Mitochondria pre-incubated with both sizes (40 and 80 nm) of silver nanoparticles were more susceptible to undergo calcium-dependent mitochondrial swelling, comparatively to control (Fig. 3), being this effect more evident for 80 nm nanoparticles. Pre-treatment with 1 μM CyA completely prevented calcium-dependent mitochondrial swelling, indicating that the decreased in absorbance was the result of the calcium-induced MPT.

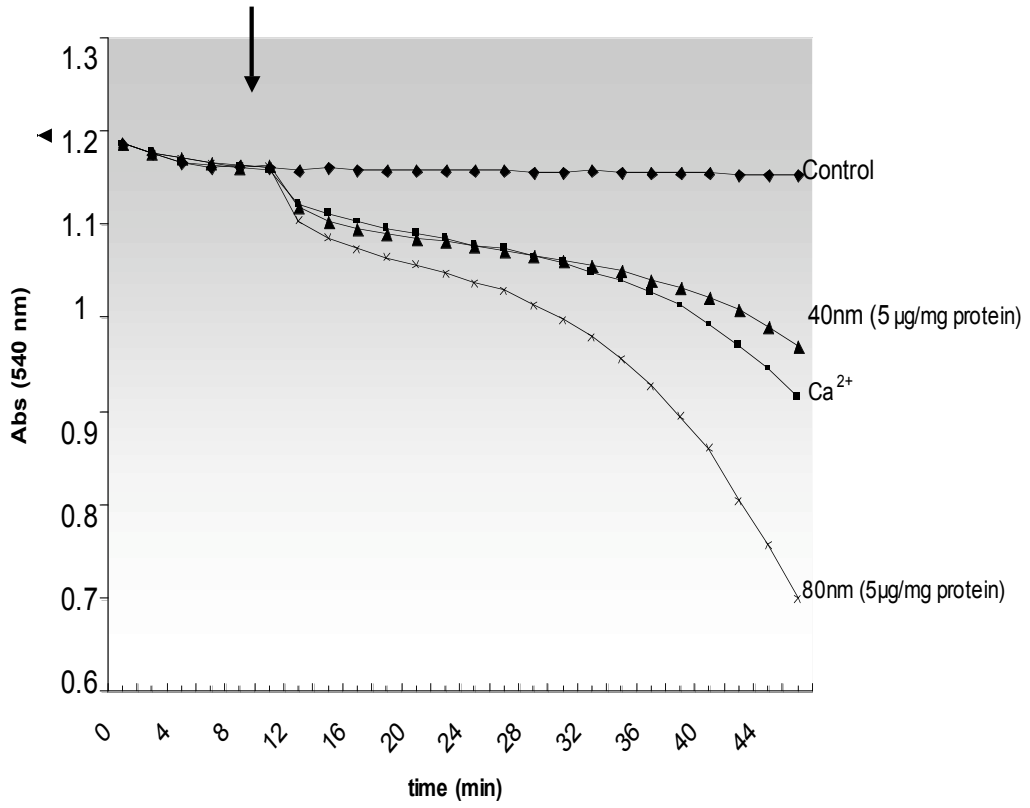

Fig. 3. Calcium-induced mitochondrial permeability transition (MPT) in liver mitochondria incubated with silver nanoparticles. Experiments were started by the addition of

mitochondria (1 mg) to 2 ml of reaction medium supplemented with 3 μM rotenone, 0.5 μg oligomycin and 5 mM succinate. MPT was induced with 20 μM or 40 μM $CaCl_2$ where indicated by the arrow. Cyclosporin A CyA (1 μM) was added to the reaction medium prior to calcium addition. The traces represent typical direct recordings of experiments performed with 10 different mitochondrial preparations.

Effects of silver nanoparticles of diferent sizes on mitochondrial calcium fluxes

Since mitochondria pre-incubated with both sizes (40 and 80 nm) of silver nanoparticles increased the susceptibility to calcium-induced permeability transition, mitochondrial calcium fluxes were evaluated. Calcium uptake by mitochondria was identical in both control and in mitochondria pre-incubated with both sizes (40 and 80 nm) of silver nanoparticles (Fig. 4). In control and treated mitochondria, calcium that was taken by mitochondria (20 or 40 μM) after energization with succinate, was retained during approximately 11 min. After this period of time, calcium was released by MPT induction, as demonstrated by pre-treatment with 1 μM CyA, which completely prevented calcium release (Fig. 4).

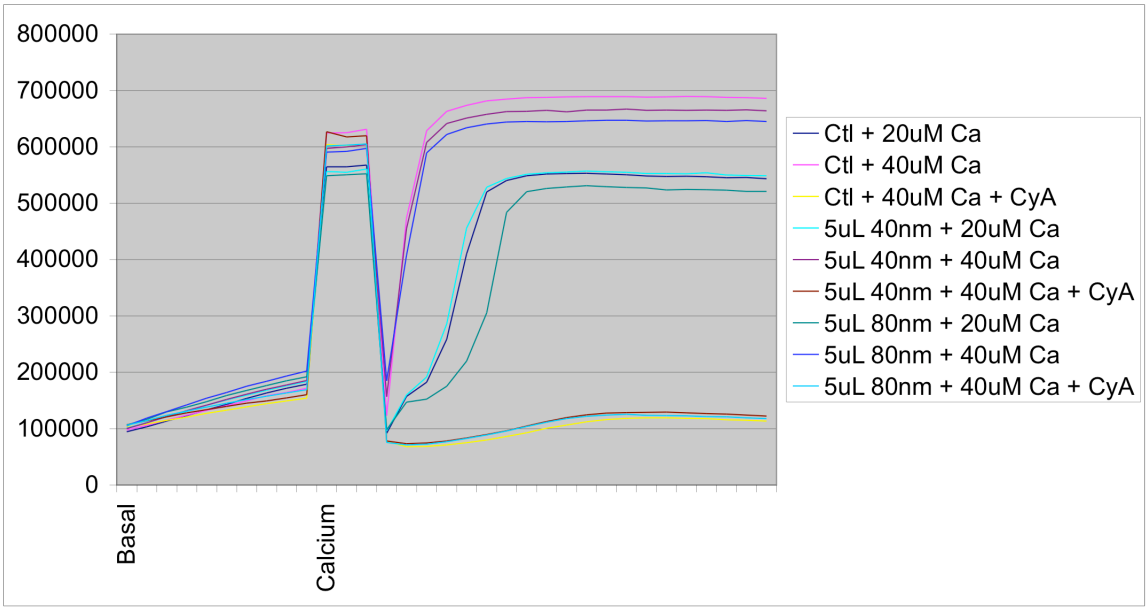

Fig. 4. Measurement of calcium fluxes in mitochondria incubated with silver nanoparticles. Mitochondria (1mg) were incubated in 2 ml of the standard incubation medium (as described in materials and methods) prior to calcium addition (10 μM). Energization was achieved with succinate 5 mM. Calcium fluxes, expressed as relative fluorescence units (RFU), were recorded for an additional 25 min. Cyclosporin A (CyA) 1μM was added to the reaction prior to calcium addition. The traces represent typical direct recordings of experiments performed with 10 different mitochondrial preparations.

Effects of silver nanoparticles of diferent sizes on Reactive Oxygen Species (ROS)

Both sizes (40 and 80 nm) of silver nanoparticles, showed no effect on the levels of ROS production by respiring liver mitochondria (Fig. 5). The addition of rotenone (a known inhibitor of the respiratory chain), did not elicit any increase in ROS production, after incubation with silver nanoparticles (Fig. 5).

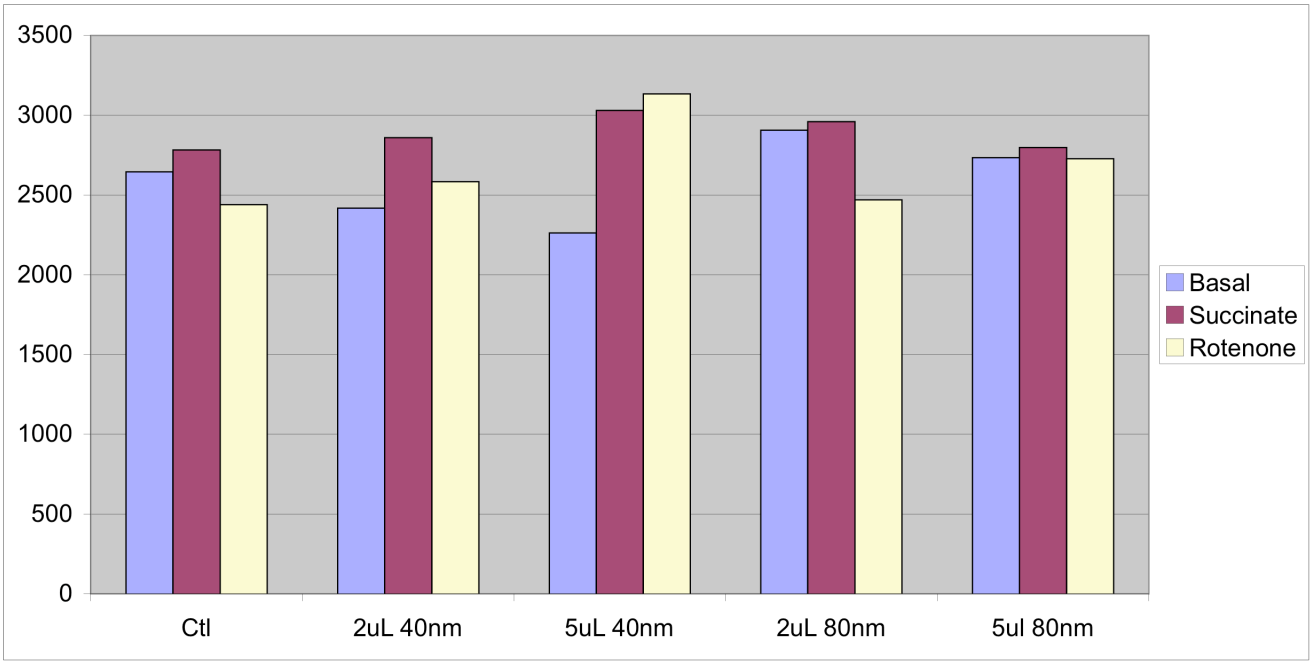

Fig. 5. Measurement of reactive oxygen species (ROS) production in mitochondria incubated with silver nanoparticles. Mitochondria (1mg) were incubated in 2 ml of the standard incubation medium, with the probe MitoSOXRed, as described in materials and methods. Energization was achieved with succinate 5 mM. ROS production, expressed as relative fluorescence units (RFU), were recorded for an additional 25 min. Data are means ± S.E.M of experiments performed with 10 different mitochondrial preparations. * indicates statistically significant difference versus control (P<0.05).

DISCUSSION

The aim of this study was to evaluate if silver nanoparticles are mitochondrial toxicants by interfering with the bioenergetic features of the mitochondria. Our results demonstrate that, in isolated rat liver mitochondria, both sizes (40 and 80 nm) of silver nanoparticles impairs the oxidative phosphorylation capacity as shown by decreased respiratory control ratio. Moreover, both sizes (40 and 80 nm) of silver nanoparticles depresses mitochondrial ΔΨ and state 3 respiration but induces stimulation of state 4 respiration. The decrease in state 3 respiratory rate indicated a loss of oxidative capacity in mitochondria incubated in the presence of silver nanoparticles. Additionally, both sizes (40 and 80 nm) of silver nanoparticles increase state 4 respiration, which paralleled the partial collapse of the ΔΨ, probably reflects an uncoupling effect of these compounds on the oxidative phosphorylation system, resulting in an increase in the permeability of the mitochondrial inner membrane to protons (proton leak).

The increase in the lag phase in mitochondria incubated with both sizes (40 and 80 nm) of silver nanoparticles, indicate an uncoupling between the oxidation of reduced substrates by the electron transport chain and the phosphorylation of ADP to ATP by the ATPsynthase. MPT induction disrupts the permeability barrier of the inner membrane, thus dissipating the membrane potential and pH gradient that together drive ATP synthesis through oxidative phosphorylation, causing impairment of the mitochondrial function.

CONCLUSION

Our results show that silver nanoparticles cause impairment of mitochondrial function, due mainly to alterations of mitochondrial membrane permeability that reflect an uncoupling effect on the oxidative phosphorylation system, being toxicity dependent on particle size. Thus, mitochondrial toxicity may have a central role in the toxicity caused by exposure to silver nanoparticles.

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